

INACTIVATION OF ALDEHYDE DEHYDROGENASE BY NITROGLYCERIN IN THE PRESENCE AND ABSENCE OF LIPOIC ACID AND DIHYDROLIPOIC ACID. IMPLICATIONS FOR THE PROBLEM OF DIFFERENTIAL EFFECTS OF LIPOIC ACID *IN VITRO* AND *IN VIVO*

ANNA BILSKA-WILKOSZ¹, MAGDALENA GÓRNY¹, MAGDALENA DUDEK²,
LESZEK NOWIŃSKI³, MAREK BEDNARSKI³, MAŁGORZATA ICIEK¹,
DANUTA KOWALCZYK-PACHEL¹, MARIA SOKOŁOWSKA-JEŻEWICZ¹, BARBARA FILIPEK²
and LIDIA WŁODEK^{1*}

¹Chair of Medical Biochemistry, Jagiellonian University, Medical College,
7 Kopernika St., PL 31-034 Kraków, Poland

²Department of Pharmacodynamics, ³Department of Pharmacological Screening, Jagiellonian University,
Medical College, 9 Medyczna St., PL 30-688 Kraków, Poland

Abstract: Lipoic acid (LA-(SS), LA) and its reduced form - dihydrolipoic acid DHLA-(SH)₂, DHLA) are synthesized mainly in the mammalian liver. In this study, we investigated *in vitro* the inactivation of yeast aldehyde dehydrogenase (ALDH) by nitroglycerin (GTN) in the presence and absence of LA and DHLA. *In vivo* studies were performed to answer the question whether LA administered jointly with GTN for 8 days will affect the ALDH activity in the rat liver. The results indicated that *in vitro* both LA and DHLA restored and protected ALDH activity against GTN-induced inactivation, while treatment of rats with LA and GTN in combination did not provide any protection against GTN-induced ALDH inhibition. In summary, the obtained results seem to confirm earlier reports indicating the differential effects of LA *in vitro* and *in vivo*.

Keywords: lipoic acid, dihydrolipoic acid, nitroglycerin, aldehyde dehydrogenase

Lipoic acid, also known as α -lipoic acid or thioctic acid (LA, LA-(SS), 1,2-dithiolane-3-pentanoic acid) and its reduced form dihydrolipoic acid (DHLA, DHLA-(SH)₂, 6,8-dimercaptooctanoic acid) are present in all prokaryotic and eukaryotic cells. The chemical structures of LA and DHLA are shown in Figure 1. LA is synthesized in mammals in the mitochondria of the liver and other tissues. It is suggested that octanoic acid is the precursor of the eight-carbon fatty acid chain of LA while cysteine is the source of sulfur. Most of LA in the body is found tightly bound to proteins by an amide linkage (lipoamide, lipoyl group) to a lysine residue (lipolysine). This lipoamide bond functions mainly as a cofactor of multienzymatic mitochondrial complexes catalyzing oxidative decarboxylation of α -ketoacids (1, 2). The lipoyl group can be oxidized and reduced by lipoamide dehydrogenase.

Apart from endogenous synthesis, LA is also absorbed from food. LA has been detected in the

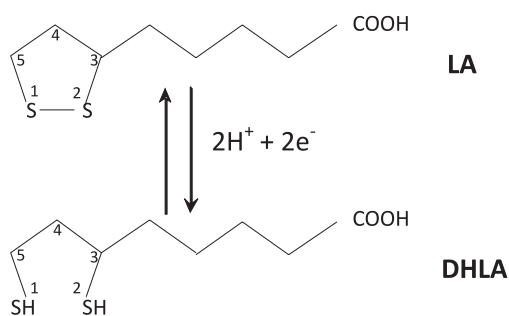


Figure 1. Chemical structures of lipoic acid (LA) and its reduced form dihydrolipoic acid (DHLA)

form of lipolysine in various natural products. The most important dietary sources of it include spinach, broccoli and meat offal (3). Lipolysine absorbed from the diet can be hydrolyzed in blood by lipoamidase. Moreover, LA has now become a common ingredient of different formulas for athletes, anti-

* Corresponding author: e-mail: mbwlodek@cyf-kr.edu.pl; phone: +48 12 4227400; fax: +48 12 4223272

aging supplements and even pet food (4). In recent years, pharmacological properties of exogenously supplemented LA have attracted attention of many researchers. It is suggested that therapeutic effects of LA supplementation can be attributed to the potent antioxidant properties of LA and DHLA.

The reductive potential of the LA/DHLA couple has been estimated at -320 mV (1) or -290 mV (5). This means that DHLA is able to reduce not only reactive oxygen and nitrogen species (ROS and RNS, respectively) but also the oxidized forms of other antioxidants, both hydrophilous, such as glutathione disulfide (GSSG) and dehydroascorbic acid (DHA), and hydrophobic, such as chromanoxyl radical of tocopherol (TOH) (6–10) (Table 1). For this

reason, DHLA is often called the antioxidant of antioxidants or a universal antioxidant (11). The oxidized form, i.e., LA has also been demonstrated to express antioxidant properties (11, 12) (Table 2).

Redox reactions are fundamental processes in metabolism of all cells. A disruption of the pro- and antioxidant balance can explain the mechanism of etiopathogenesis and progression of many, apparently distant pathological states. This is the reason for an increasing significance of antioxidants in medicine and more common use of the term “oxidative stress” in medical sciences. However, since the activity of LA and DHLA as direct scavengers of ROS/RNS was confirmed mostly by *in vitro* experimental studies, some authors question the efficacy

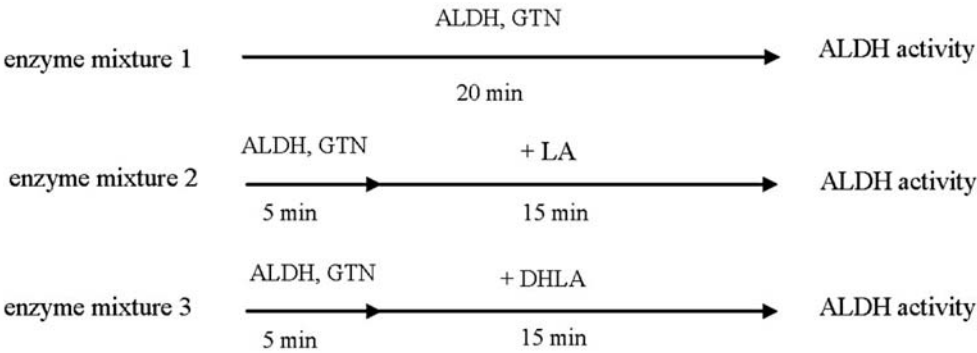
Table 1. The values of standard biological redox potentials (E_0') of selected redox systems

System	E_0' (V)	Reference
Acetic acid/acetaldehyde	-0.60	(11)
Acetylo-CoA/acetaldehyde	-0.41	(11)
NAD/NADH + H ⁺	-0.32	(12, 14)
LA/DHLA	-0.29	(5, 12, 13)
GSSG/GSH	-0.24	(12)
Cystine/cysteine	-0.22	(12, 15)
Acetaldehyde/ethanol	-0.20	(11)
FAD/FADH ₂	-0.06	(11, 14)
Dehydroascorbic acid/ascorbic acid	0.08	(11, 12)
Chromanoxyl radical of tocopherol/tocopherol	0.48	(13, 14)
O ₂ /H ₂ O	0.82	(14)
H ₂ O ₂ /O ₂ ^{•-}	0.87	(14)
NO ⁺ /NO [•]	1.21	(15)
H ₂ O ₂ /H ₂ O	1.32	(14)
ONOO ⁻ /NO ₂ [•]	1.40	(15)
•OH, H ⁺ /H [•] O	2.31	(15)

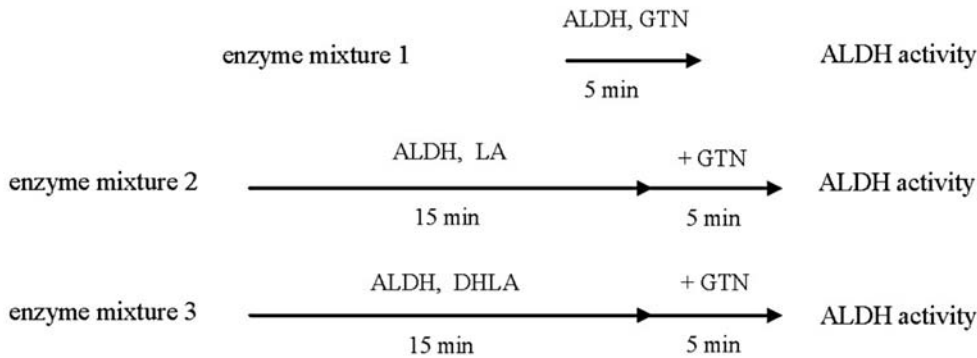
Table 2. The scavenging capacity of LA and DHLA for the selected ROS and RNS.

ROS/RNS	LA	DHLA	Scavenging capacity	Reference
O ₂ ^{•-}	no	yes	-	(11)
	no	no	-	(12)
H ₂ O ₂	yes	yes	LA < DHLA	(11)
	no	yes	-	(12)
•OH	yes	yes	LA < DHLA	(11)
			LA = DHLA	(12)
ONOO ⁻	yes	yes	LA < DHLA	(11, 12)
NO [•]	yes	yes	LA < DHLA	(11, 12)

Series I was prepared as follows:



Series II was prepared as follows:



Scheme 1. Design of the experiment investigating yeast ALDH activity *in vitro*. Two series of enzyme mixtures were prepared. The final concentration of ALDH was 0.3 mg/mL in all experiments. The concentration of thiols (LA and DHLA) in the enzyme mixtures was 1 mM

of the supplemented nonprotein-bound LA as a physiological antioxidant and underline the problem, overlooked until recently, related to the differential effects of LA *in vitro* and *in vivo* (13, 14). Our research paper is a modest contribution to the ongoing discussion on this issue.

In the present study, we investigated *in vitro* the inactivation of yeast aldehyde dehydrogenase (ALDH) by nitroglycerin (glycerol trinitrate; GTN) in the presence and absence of LA and DHLA. Moreover, *in vivo* studies were performed to answer the question whether LA administered jointly with GTN will affect the ALDH activity in the rat liver.

The genes encoding ALDHs were divided into several families and subfamilies. It should be noted that sequence comparison demonstrated that both

yeast and mammalian ALDH isozymes contained -SH groups that could form a wide variety of oxidation products (15).

EXPERIMENTAL

Reagents

In this study, the formulations Thiogamma and Nitracor were used, which contain LA and GTN, respectively, as pharmacologically active substances. Thiogamma was obtained from Hexal® AG, (Holzkirchen, Germany). Nitracor was purchased from LEK S.A. and Pliva Kraków S.A. (Poland). Purified yeast ALDH, as well as DHLA, EDTA, Folin-Ciocalteu reagent, 4-methylpyrazole, NAD, propionaldehyde and rotenone were provided

by Sigma-Aldrich Chemical Company ((Poznań, Poland). All the other reagents were of analytical grade and were obtained from Polish Chemical Reagent Company (POCH, Gliwice, Poland).

Animals

Animal experiments were conducted in accordance with the guidelines for animal experiments of Animal Research Committee and were approved by the Jagiellonian University Ethic Committee. The study was carried out on male Wistar rats using the modified experimental design of Dudek et al. (16, 17). The animals were divided randomly into three groups of 8 animals each. The first group was treated with 0.9% NaCl (0.6 mL, three times a day, *s.c.*)

for 8 days (control group). The second group was treated with GTN (30 mg/kg b.w., *s.c.*, divided into three doses) for 8 days. The third group was treated with GTN and LA jointly for 8 days (GTN 30 mg/kg b.w., *s.c.*, divided into three doses and LA 100 mg/kg b.w., *i.p.*, divided into two doses, respectively).

Animals were sacrificed on the 9th day by cervical dislocation and the livers were excised, washed in 0.9% NaCl, placed in liquid nitrogen and stored at -70°C until ALDH activity test was performed.

Methods

Preparation of liver homogenates

The frozen livers were weighed and homogenates were prepared by homogenization of 1 g of the tissue in 4 mL of 0.1 M phosphate buffer, pH 7.4 using an IKA-ULTRA-TURRAX T8 homogenizer.

Determination of ALDH activity in the rat liver homogenate

The assay mixture contained liver homogenate, sodium phosphate buffer (pH 8.2), NAD, EDTA, 4-methylpyrazole and rotenone. The reaction was initiated by the addition of propionaldehyde as a substrate. 4-Methylpyrazole was added to inhibit alcohol dehydrogenase, and rotenone to inhibit mitochondri-

Table 3. The effect of GTN administered in combination with LA for 8 days on the ALDH activity in the rat liver.

Treatment	ALDH [U/mg protein]
Control group	45.28 ± 10.85
GTN group	21.12***± 3.87
GTN + LA group	26.25*** ± 5.38

***Differences significant at $p < 0.001$ in comparison to control value. The activity of ALDH was calculated by using the molar extinction coefficient of reduced NAD of $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ at 340 nm. Specific activity of the enzyme was expressed as nmol NADH/min (U)/mg protein.

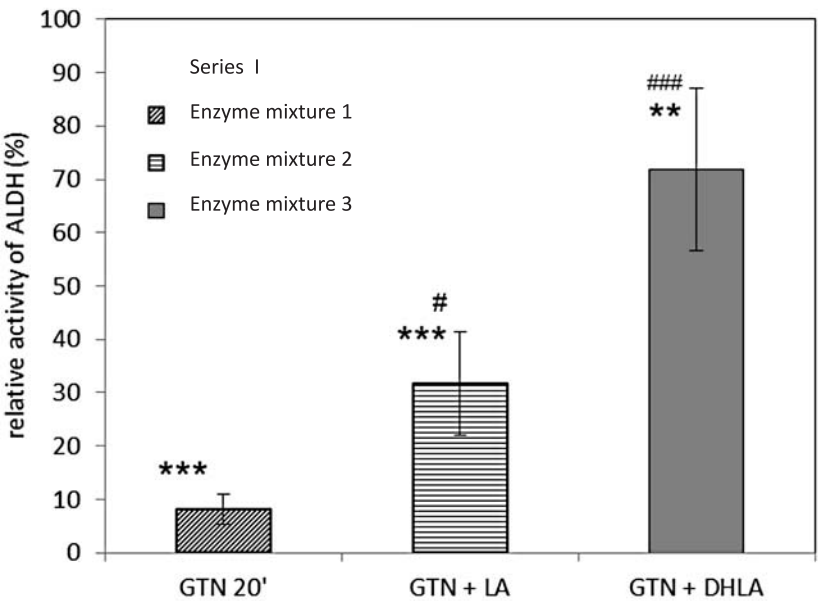


Figure 2. The LA and DHLA-induced restoration of GTN-inhibited ALDH activity *in vitro*. The ALDH activity was assayed with propionaldehyde (8 μM) as a substrate by measuring the reduction of NAD (1 mM) at 340 nm. Data are presented as a percentage relative to the control (100%) (without GTN and thiols). Data are shown as the mean ± SD. Significant vs. control sample: ** $p < 0.01$; *** $p < 0.001$. Significant vs. GTN sample: # $p < 0.05$, ### $p < 0.001$

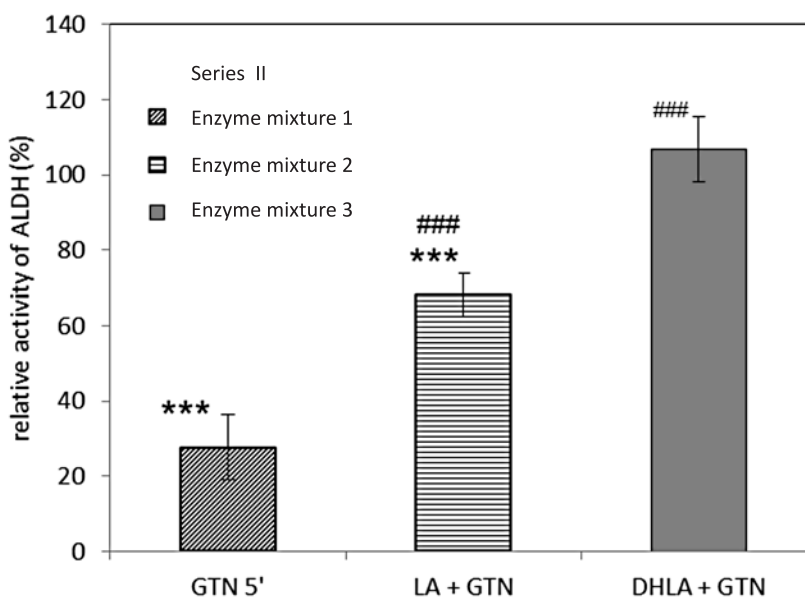


Figure 3. The LA and DHLA-induced prevention of ALDH inactivation by GTN *in vitro*. The ALDH activity was assayed with propionaldehyde (8 μ M) as a substrate by measuring the reduction of NAD (1 mM) at 340 nm. Data are presented as a percentage relative to the control (100%) (without GTN and thiols). Data are shown as the mean \pm SD. Significant vs. control sample: *** $p < 0.001$. Significant vs. GTN sample: ## $p < 0.01$; ### $p < 0.001$.

al NADH oxidase. The blank sample in which the homogenate was omitted was run simultaneously. The activity of ALDH was calculated using the molar extinction coefficient of NADH of 6.22 $\text{mM}^{-1}\text{cm}^{-1}$ at 340 nm with the use of a modified protocol published earlier (18, 19). Specific activity of the enzyme was expressed as nmol of NADH produced per 1 mg of protein per 1 min. The protein content was measured using the method of Lowry et al. (20).

Determination of yeast ALDH activity

The final concentration of ALDH was 0.3 mg/mL in all experiments. The concentration of thiols (LA and DHLA) in the enzyme mixtures was 1 mM. Two series of enzyme mixtures were prepared (Scheme 1). All enzyme-containing mixtures were incubated at a temperature of 25°C.

Series I was prepared as follows:

Enzyme mixture 1. ALDH was preincubated with 0.1 mM GTN in 50 mM sodium phosphate buffer (pH 8) for 20 min.

Enzyme mixture 2. ALDH was preincubated with 0.1 mM GTN in 50 mM sodium phosphate buffer (pH 8) for 5 min, then LA was added and the mixture was incubated further for 15 min.

Enzyme mixture 3. ALDH was preincubated with 0.1 mM GTN in 50 mM sodium phosphate

buffer (pH 8) for 5 min, then DHLA was added and the mixture was incubated further for 15 min.

Series II was prepared as follows:

Enzyme mixture 1. ALDH was preincubated with 0.1 mM of GTN in 50 mM sodium phosphate buffer (pH 8) for 5 min.

Enzyme mixture 2. ALDH was preincubated with LA in 50 mM sodium phosphate buffer (pH 8) for 15 min, then 0.1 mM GTN was added and the mixture was incubated further for 5 min.

Enzyme mixture 3. ALDH was preincubated with DHLA in 50 mM sodium phosphate buffer (pH 8) for 15 min, then 0.1 mM GTN was added and the mixture was incubated further for 5 min.

The assay mixture (1 mL) containing sodium phosphate buffer (pH 8.2), propionaldehyde and NAD was pipetted into a cuvette. The reaction was initiated by the addition of the indicated enzyme mixture to the cuvette and absorbance change at 340 nm was monitored for 2 min at 25°C to calculate the rate of NADH production and to compare it with the sample containing only ALDH without GTN (control sample). Data are presented as a percentage relative to control (100%).

Statistical analysis

All statistical calculations were carried out with the STATISTICA 10.0 computer program

using a one-way ANOVA followed by the Tukey *post-hoc* test. Data from *in vitro* studies are presented as the mean \pm SD of 3-4 independent experiments in several repetitions. Data from *in vivo* experiments are given as the mean \pm SD for each group of animals. For all data, the values of $p < 0.05$ were considered to be statistically significant.

RESULTS

Activity of ALDH in the rat liver homogenates

As shown in Table 3, the activity of ALDH in the rat liver of the second group (GTN) and the third group (GTN + LA) was statistically significantly decreased vs. control group (first group).

Activity of yeast-derived ALDH

The results obtained in series I are presented in Figure 2. The experiment indicated that 20-min incubation with GTN induced a decrease in the ALDH activity to 8.2% (mixture I) as compared to the control (100%). A five-minute preincubation of the enzyme with 0.1 mM GTN and then a 15-min incubation with 1 mM thiols: LA or DHLA (mixture 2 and 3, respectively) caused a decrease in ALDH activity to 31.6% and 71.9%, respectively, compared to the control (100%). This indicates that both 1 mM thiols (LA and DHLA) were able to restore ALDH activity inhibited by GTN, but DHLA was more effective than LA.

The results obtained in series II are presented in Figure 3. The experiment demonstrated that a 5-min incubation with GTN lowered ALDH activity to 27% (mixture 1) compared to the control (100%). When the samples of ALDH were preincubated with 1 mM LA or DHLA (mixture 2 and 3, respectively) for 15 min and then with 0.1 mM GTN for 5 min, the ALDH activity changed to 68.1% and 106.9%, respectively, compared to the control (100%).

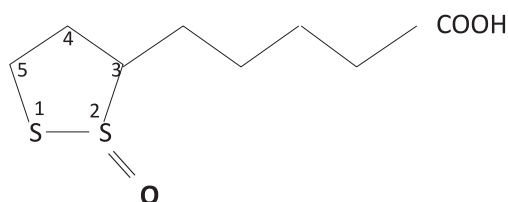


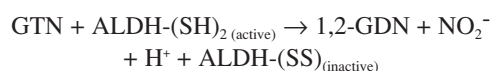
Figure 4. Chemical structure of β -lipoic acid (β -LA). β -LA is a biological oxidation product of LA, which was proven in *in vitro*, but not *in vivo* studies (12, 27).

This indicates that both 1 mM thiols (LA and DHLA) were able to protect ALDH against the inhibitory effect of GTN, but DHLA was more effective than LA.

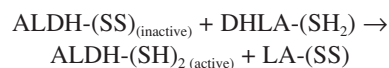
DISCUSSION AND CONCLUSIONS

In line with literature data, the present studies demonstrated that GTN inhibited ALDH activity both *in vivo* and *in vitro*. Already in 1985, Towell et al. (21) observed an 88% inhibition of erythrocytic ALDH in GTN-treated patients. Wenzel et al. (22) indicated that GTN was a potent inhibitor of both purified yeast ALDH as well as ALDH in isolated heart mitochondria from *in vivo* GTN-treated rats. The next study of the same research group demonstrated that GTN inhibited ALDH activity in circulating white blood cells (WBCs) of healthy volunteers (23). The studies of Beretta et al. (24) showed that GTN blocked ALDH activity in isolated rat liver mitochondria, and also of recombinant human ALDH.

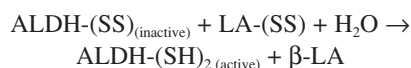
It is currently known that GTN is an inhibitor of ALDH (i.e., causes the loss of dehydrogenase activity) because it serves as a substrate for its reductase activity. This means that ALDH catalyzes the formation of glyceryl dinitrate (GDN) (1,2-GDN/1,3-GDN \sim 8 : 1) and nitrite (NO_2^-) from GTN. The electrons for this reduction are provided by cysteine-thiols at the active site of ALDH that are converted to an intramolecular disulfide bond, which leads to a drop in the catalytic activity of the enzyme (24–26). A simplified scheme of this process can be expressed as:



The present results obtained *in vitro* support the role of DHLA both in the restoration of ALDH activity after inhibition by GTN and in the protection of the enzyme against the inhibitory effect of GTN. ALDH activity was not inhibited by GTN if DHLA was added prior to GTN. Also, DHLA largely restored the inhibited ALDH activity if added after GTN. It was confirmed by studies of other authors. Namely, regeneration of the oxidized $-\text{SH}$ groups of ALDH could be achieved *via* an exchange with $-\text{SH}$ groups of DHLA (22, 24) what, in a simplified way, can be expressed as:



It is interesting and surprising that under *in vitro* conditions also LA prevented inactivation of the enzyme and had an impact on ALDH already inhibited by GTN. Since this is the first study examining the effect of GTN on the ALDH activity in the presence of the oxidized form of DHLA, i.e. LA, this result is difficult to explain because of too few data. *In vitro* studies revealed that LA was capable of scavenging numerous ROS and RNS, which indicated that despite being in its oxidized form LA behaved as a reducing agent (antioxidant). β -Lipoic acid (β -LA) (Fig. 4) is a biological oxidation product of LA, as demonstrated only by *in vitro* studies (12, 27, 28). It means that reduction process of the oxidized –SH groups of ALDH, in a simplified form, can be expressed as:



Is it possible? Further studies are necessary to verify this undoubtedly intriguing hypothesis.-

On the other hand, the *in vivo* experiments indicated that treatment of rats with LA and GTN in combination did not provide any protection against GTN-induced ALDH inhibition. This is in contrast with the results of Wenzel et al. (23), who reported that administration of a single sublingual GTN dose decreased ALDH activity in WBCs in healthy volunteers and that these effects were prevented by oral LA pretreatment 15 min before GTN.

This difference could possibly be due to a number of factors, including different experimental conditions. In our opinion, the level of oxidative stress is the core of the problem since oxidative stress was undoubtedly much lower after GTN administration at a single dose compared with chronic GTN treatment (8 days). The sulfhydryl groups of cysteine residues (Cys-SH) in proteins (P-SH) can form a wide variety of oxidation products, including reversible oxidation states of disulfide bonds (P-SS) and sulfinic acid (P-SOH), and the irreversible higher oxidation states of sulfinic acid (P-SO₂H) and sulfonic acid (P-SO₃H) (15, 29). Thus, the plausible proposal is that under our experimental conditions *in vivo* the sulfhydryl groups of cysteine residues at the active site of ALDH can be irreversibly oxidized to sulfinic acid (P-SO₂H) and sulfonic acid (P-SO₃H) that cannot be reduced by LA and DHLA, but in experimental conditions *in vivo* described in the above-mentioned paper by Wenzel et al. (23) the activity of the enzyme decreased due to reversible oxidation of sulfhydryl groups of cysteine residues in ALDH to disulfide bonds that can be reduced by LA and DHLA.

It should be mentioned that recent experimental and clinical studies indicate a beneficial effect of exogenous LA in many diseases accompanied by oxidative stress (30–36) and simultaneously, the ability of LA and DHLA to function as physiological antioxidants is questioned (13, 14). It means that the mechanism of pharmacological actions of LA *in vivo* still remains undiscovered. This issue definitely requires further studies the more so that searching for the mechanisms of pharmacological actions of LA is important from the point of view of both basic research and its practical applicability.

In summary, the obtained results seem to confirm earlier reports indicating the differential effects of LA *in vitro* and *in vivo*.

Conflict of interest

The authors do not have any conflict of interest regarding this manuscript.

Acknowledgments

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